# Differential Regulation of RNA Levels of Gibberellin Dioxygenases by Photoperiod in Spinach[1]

## Dong Ju Lee and Jan A.D. Zeevaart*

Department of Energy-Plant Research Laboratory (D.J.L., J.A.D.Z.), and Department of Plant Biology (J.A.D.Z.), Michigan State University, East Lansing, Michigan 48824–1312

Previous work with spinach (*Spinacia oleracea*) has shown that the level of gibberellin (GA) 20-oxidase is strongly up-regulated by long days (LD). In the present work, the effect of photoperiod on expression of other GA dioxygenases was investigated and compared with that of GA 20-oxidase. Two GA 2-oxidases and one GA 3-oxidase were isolated from spinach by reverse transcription-polymerase chain reaction with degenerate primers and by 5′- and 3′-rapid amplification of cDNA ends. As determined by high-performance liquid chromatography with on-line radioactivity detection, the *SoGA3ox1* gene product catalyzed 3β-hydroxylation of $GA_9$ to $GA_4$ and $GA_{20}$ to $GA_1$. The *SoGA2ox1* and the *SoGA2ox2* gene products catalyzed 2β-hydroxylation of $GA_9$ to $GA_{51}$ and $GA_{20}$ to $GA_{29}$. The product of $GA_{20}$ metabolism by SoGA3ox1 was identified as $GA_1$ by gas chromatography-mass spectrometry, whereas the products of $GA_1$ and $GA_{20}$ metabolism by SoGA2ox1 and SoGA2ox2 were identified as $GA_8$ and $GA_{29}$, respectively. SoGA2ox1 also metabolized $GA_{53}$ to $GA_{97}$. The levels of *SoGA20ox1* transcripts were greatly increased in all organs tested in LD conditions, but the levels of *SoGA3ox1* transcripts were only slightly increased in blades and petioles. A decrease in the levels of the *SoGA2ox1* transcripts in young leaves and tips in LD conditions is opposite to the expression pattern of the *SoGA20ox1*. Expression of *SoGA20ox1* in petioles and young leaves was strongly up-regulated by a supplementary 16 h of light, but the levels of *SoGA3ox1* and *SoGA2ox1* transcripts did not change. It is concluded that regulation and maintenance of $GA_1$ concentration in spinach are primarily attributable to changes in expression of *SoGA20ox1*.

Long-day (LD) rosette plants, such as spinach (*Spinacia oleracea*), grow vegetatively and do not produce a stem when grown under short-day (SD) conditions. Upon transfer to LD conditions, stem elongation and flowering take place. In spinach, LD-induced stem elongation is dependent on gibberellin (GA)-regulated processes. There is considerable evidence that GA biosynthesis increases in rosette plants in LD conditions (Talon and Zeevaart, 1990; Talon et al., 1991). The major endogenous GAs of spinach belong to the early-13-hydroxylation pathway: $GA_{53}$, $GA_{44}$, $GA_{19}$, $GA_{20}$, $GA_1$, $GA_8$, and $GA_{29}$ (Fig. 1; Talon et al., 1991), of which only $GA_1$ is active per se (Zeevaart et al., 1993). Several steps of the GA biosynthetic pathway are regulated by photoperiod. For example, the biosynthesis of *ent*-kaurene, an early step in the GA biosynthetic pathway, is enhanced by LD (Zeevaart and Gage, 1993). The later steps of sequential oxidation and elimination of C-20 of $C_{20}$-GAs ($GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$), catalyzed by GA 20-oxidase, are also increased when spinach plants are transferred from SD to LD conditions (Wu et al., 1996).

3β-Hydroxylation is the final step to active GAs, converting $GA_9$ and $GA_{20}$ to $GA_4$ and $GA_1$, respectively. Genes encoding 3-oxidases have been isolated from several species, and their expression and regulation have been studied (Chiang et al., 1995; Lester et al., 1997; Cowling et al., 1998; Yamaguchi et al., 1998; Itoh et al., 1999, 2001; Rebers et al., 1999). GA 2-oxidases introduce a 2β-hydroxyl group to biologically active GAs and deactivate $C_{19}$-GAs, such as $GA_1$, $GA_4$, $GA_9$, and $GA_{20}$ (Lester et al., 1999; Thomas et al., 1999; Elliott et al., 2001). Both GA 3-oxidase and GA 2-oxidase can, therefore, directly regulate the levels of the active GAs, $GA_1$ and $GA_4$, and their immediate precursors, $GA_{20}$ and $GA_9$, respectively (for reviews, see Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000).

As shown in earlier work with spinach, transfer of plants from SD to LD causes an increase in all GAs of the early-13-hydroxylation pathway, with $GA_{20}$, $GA_1$, and $GA_8$ showing the largest increases (Wu et al., 1996). The increase in $GA_{20}$ can be explained as being attributable to increased expression of the gene encoding GA 20-oxidase, *SoGA20ox1*. However, to what extent the levels of $GA_1$ and $GA_8$ are determined by photoperiodically induced changes in expression of GA 3-oxidase and GA 2-oxidase is not known. In this study, we describe the molecular cloning of a GA 3-oxidase and two GA 2-oxidases from spinach and the regulation of their expressions by photoperiod in comparison with that of GA 20-oxidase. Our results indicate that expression of GA
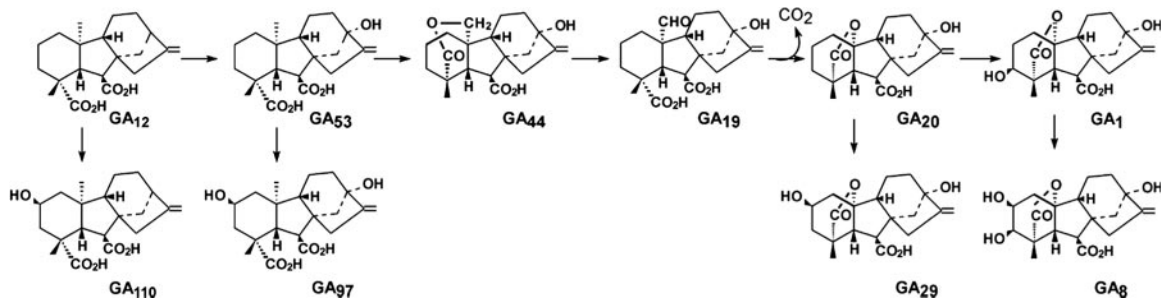
**Figure 1.** The early-13-hydroxylation pathway from $GA_{12}$ to $GA_8$ of GA biosynthesis and deactivation in spinach. $GA_{53}$ is converted to $GA_{20}$ by GA 20-oxidase via $GA_{44}$ and $GA_{19}$. $GA_{20}$ can be converted to $GA_1$ by GA 3-oxidase. $GA_{12}$, $GA_{53}$, $GA_{20}$, and $GA_1$ can be deactivated by $2\beta$-hydroxylation to $GA_{110}$, $GA_{97}$, $GA_{29}$, and $GA_8$, respectively.

20-oxidase1 and GA 2-oxidase1 is affected more by the photoperiod than is expression of GA 3-oxidase1 and GA 2-oxidase2.

## RESULTS

### Cloning of GA 3-Oxidase and GA 2-Oxidase cDNAs

Reverse transcription (RT)-PCR reactions were performed with total RNA as a template isolated from spinach grown under SD conditions. All combinations of degenerate primers were used in the PCR reactions with first-strand cDNA as a template. The combination of degenerate primers JZ418 and JZ432 (Table I) yielded a 521-bp fragment of GA 3-oxidase (data not shown). The sequence of this RT-PCR product was homologous to the GA 3-oxidase genes of Arabidopsis (Chiang et al., 1995) and pea (*Pisum sativum*; Lester et al., 1997). 5'- and 3'-RACE were performed with sequence-specific primers (JZ445 and JZ444) using the SMART RACE cDNA Amplifi-

cation Kit (BD Biosciences Clontech, Palo Alto). The coding region of the *SoGA3ox1* gene was obtained with primers JZ509 and JZ510 (Table I). The full-length cDNA clone of *SoGA3ox1* (1,612 bp; accession no. AF506280) has an open reading frame (ORF) of 1,149 bp, encoding a putative protein of 382 amino acids with a 223-bp 5'-untranslated sequence and a 240-bp 3'-untranslated sequence. The predicted protein molecular mass is 42 kD, with an pI of 8.39 (Fig. 2). Northern-blot analysis showed two sizes of transcripts of the *SoGA3ox1* gene in shoot tips around 1.6 and 2.0 kb (data not shown). The longer transcript is immature because it contains one intron of 361 bp. This exon/intron structure is conserved at the same position as in other *GA3ox* genes (Lester et al., 1997). When compared with other GA 3-oxidases, the deduced amino acid sequence of *SoGA3ox1* belongs to the same class of enzymes and contains certain conserved amino acid sequences, such as His-227, Asp-229, and His-284, that may bind iron (Thomas et al.,

**Table I.** *Primers used for the amplification of gibberellin dioxygenase genes from spinach*

Y (C/T), N (A/T/G/C), R (A/G), S (C/G), M (A/C), H (A/T/C), D (A/G/T), K (G/T), V (A/C/G), B (C/G/T), W (A/T).

| Purpose | Primer | Primer Sequence (5'–3') | Gene Amplified | Orientation |
|---|---|---|---|---|
| RT-PCR | JZ254 | TYGGRGARCAYCANGAYCC | *GA2ox1 and -2* | S |
| RT-PCR | JZ258 | CCDSCRAARTADATCATHGA | *GA2ox1* | AS |
| 3'-RACE | JZ282 | TTTCATACTCGTTGGGGACTC | *GA2ox1* | S |
| 5'-RACE | JZ298 | CACCTGCAAAGAGTCCCCAACGAGT | *GA2ox1* | AS |
| Coding region | JZ300 | AAACCCGGGATGGTGGTGTTATCTCATGTT | *GA2ox1* | S |
| Coding region | JZ301 | CATCTCGAGTTAGTTATGATTTCTCTTTAC | *GA2ox1* | AS |
| QRT-PCR | JZ385 | ACCAAGGCCTGAGGCGTTTGAAG | *GA2ox1* | S |
| QRT-PCR | JZ386 | AACCTAGATTTGTAAGCTGAT | *GA2ox1* | AS |
| QRT-PCR | JZ389 | ATGGAAGCTSCDGGWATYCA | *Actin* | S |
| QRT-PCR | JZ390 | TTARAARCAYTTYCTGTGCAC | *Actin* | AS |
| RT-PCR | JZ342 | TCATCWGGWGGRACWGARAYCCA | *GA2ox2* | AS |
| 5'-RACE | JZ375 | CAAGCCATCAGGCATACAAATC | *GA2ox2* | AS |
| 3'-RACE | JZ574 | CAAACAATTCAACAACCAAAGCA | *GA2ox2* | S |
| Coding region | JZ582 | CGCGCTCGAGTCATTGGTCATTAATACGTCTC | *GA2ox2* | AS |
| Coding region | JZ619 | CGCGGATCCCCATGGTGGTAGCTTCTCCA | *GA2ox2* | S |
| RT-PCR | JZ418 | AARCTYATGTGGTMYGARGGVTTYAC | *GA3ox1* | S |
| RT-PCR | JZ432 | GAHGGKGGNCCATAKARATARGC | *GA3ox1* | AS |
| 5'-RACE | JZ445 | TGTAATTATTAGGCCAAAGTTGACGAGCAT | *GA3ox1* | AS |
| 3'-RACE | JZ444 | GCAATACGACGGGGCTCCAAGTGT | *GA3ox1* | S |
| Coding region | JZ509 | GCGCCCCGGGATGCCATCTAGGCCATCTC | *GA3ox1* | S |
| Coding region | JZ510 | GCGCGTCGACTTAACCAAATACAGCAAGG | *GA3ox1* | AS |

```
SoGA3ox1    1  MPSRPSRVVKEQHPTKKSPLDLESLNELPDSFANGSFEDPCSIDNPSGYGPDSVPVIN----LODPQAQQLVGLACRSNG
NtGA3ox     1  MPSR--ISDSFR-AHSOKHLDLNSIKELPDSHAWTSSNDYPS-ENSCNFESIPVIDLDNYINNNNINVLEHIGCACKKNG
StGA3ox     1  MPSR--ISDSCRPHHSOKHFDLNSIKELPDSHAWTSSNDOXTOENSCNFESIPVIDLDNNNNNNNNILDHIGCACKKNG
PsGA3ox     1  MPDSLSEAYRAHPVHVNHKHFDFNSLOELPDSYNDTHLDDHTLIDSSNNIMKESTTTVP--VIDLNDPNASKLIGLACKTNG
AtGA3ox     1  MSSDT-LSDVFRSHPIHIPLSNPPDFKSLPDSYTDTPKDDLLFSASASDETLPLIDLS-------DIHVATLVGHACTTNG
CmGA3ox     1  M---ATTOTHHSVHITHKDHDLDDSLOVLPDSYQWVQPDLFPSLOOHSTPLSDTLPLID----LSLPDAPDLIGKALRTNG
CmGA23ox    1  MDKL-------PAVGLLTPLDLNTVEAVEFSHVNPHLDESSOKFKSDKTVSIPVVDFN------DDNVLELIGKACEENG
OsGA3ox     1  MP-------TPSHLKNPLCFEFRAARRVBETHANPGLDDHPVVDGGGGGGGEDAVPVVD----VRAGDAAARVARAAEQNG

SoGA3ox1   77  VFGVTNHGIOKSLLDDIEAAGKSLDALEVNOKLKAARSSCGVTGYGHAGLSSFPEKRMWSEGFTIL-GSPLDHAROLWB-
NtGA3ox    77  AEQLINIINISERLLODIELAGKSLDSLEMOCKLKAARSPEGVTGYGVARISSPDSKLMWSDGFTIV-GSPLEIIAROTWP-
StGA3ox    79  AEQLINILSISEKLLODIEVAGKTLDSLEMOCKLKAARSPDGVTGYGVARISSPDSKLMWSEGFTIV-GSPLEIIAROLWE-
PsGA3ox    79  VYQVMANHGIPLSLLEDIOWLGOTLLFSLESHOKLKAFRSPDGVSGYGIARISPDPKLMWYEGFTIV-GSPLDHFRDLWE-
AtGA3ox    73  AEQITNHGVPSRDLDDIEFLTGSLDRLEVORKLKAARSENGVSGYGHAARSENGVSGYGVARISPDHKKMWSDGFTVI-GSPLHDFRKLWB-
CmGA3ox    74  SFQLINHGVPLSLLIHAAOSSAIDLDALETPYKLKASRTPGTFPGYGIARINSRPEKRMWSDSFTIM-GSPLEHFRHLWP-
CmGA23ox   68  MEQLINHGIPKTLLTAETEEVARCPDALLFKSOKMWTLNVPGTANGYCMARLTKHHDKMMWWSEGFTVI-GSPVDDFKKLWB-
OsGA3ox    70  AELLVGHGVPAALLSRVEERVARVGSLDLASEGMRAVRGPGEPCGYGGPPLSSFDSKLWMSGYTFSPSSLRSELRRLWBK

SoGA3ox1  155  --NNYNKPCDIILKYOKEMNOLAKKLMOLVVGSLGIS-NODIMNWA---DLLEGANGAMOLNSYPIRPDPNRAMGLAAHT
NtGA3ox   155  --HDYOKPCVIEEYEREMEKLAGRLNWLMLGSLGIT-KEEVKWAVCPKGESKGGSAALOLNSYPACPDPDRAMGLAAHT
StGA3ox   157  --KDYNKPCGEVIEYEKEMEKLAGRTLDSLEMOCKLKAARSPDGVTNICDDLHILSNGLYPSVLHRAVVNRTRYR-LSVAYIYGPESN
PsGA3ox   157  --ODYTFRPCGILVVCGVDETKKLAGTLMCLMLDSLGIT-KEDIKWAG-SKAOFEKACAALOLNSYPSCPLDDHAMGLAPHT
AtGA3ox   151  --SHHLKYCEIIPEYEEHMOOKLAAKLMWFALGSLGVE-EKDIOWAG-PNSDFOGTOAAIOLNOXEKGPKPDPRAMGLAAHT
CmGA3ox   152  --HDYSKRCDIMAEYDRELKSLCGRLNWIALSELGIT-REDINWAG-PNEDFKTCNAATOLNBYPACPDPDRAMGLAAHT
CmGA23ox  146  --SDYOPPCDKVEEYOLKMKALADKLISLIFKYLGISDEEMVKKLSYIDPATGKPHLALRLNSBICPIPDSKWMGLAAHT
OsGA3ox   150  SGDDYLLPCRVMPREHKEWRRLADEDLRLFLRALGLT-GEEVAGVEAERRIGERMTATVHNAYCRCPERRALGIIAHT

SoGA3ox1  229  DSTLLAILHQSNTTGLQVFRER-SGWVTVPFISGGAVLNIGDLIHILSNGRYPSVHRAVVNROHR-LSVAYIYGEASG
NtGA3ox   232  DSTILAILHCNNTSGLQVFKEG-SGWVTVPFEFPGALVNVGDLIHILSNGLYPSVLHRAVVNRTRHR-LSVAYIYGPBSR
StGA3ox   234  DSTILAILHCNNTSGLQVYOEG-NGWITVPFIPGALVVNNICDDLHILSNGLYPSVLHRAVVNRTRYR-LSVAYIYGPESN
PsGA3ox   233  DSTFLAILSONDISGLQVNREG-SGWITVPFLOGGLVVNVGDLFYILSNGLYPSVLHRVLVNDRTROR-FSVAYIYGPESN
AtGA3ox   227  DSTLMAILLYONNTAGLQVFRDD-VGGVTAPEVPGSLVVNVGDLIHILTNGIFRSVLHRAVVNHVRSR-FSMAYIWGPPSD
CmGA3ox   228  ETTLLAVILHCNNTRGLCILREG-ROWVTVELVAGALMWOVGMOIVSNGLYKSPFEAWWNRTOKR-LSVAYIFVPBEN
CmGA23ox  224  ETSLFOMLHOARREGLCILNEK-EGGLPLARRRDAILTNICDDGOILSNGRFHSVPHRVMIRETEKTITMSMDWFFHBGH
OsGA3ox   229  ESGFFLPCRVMPREHKEWRRIADEDLRLFLRALGLT-GEEVAGVEAERRIGERMTATVHNAYCRCPERRALGIIAHT

SoGA3ox1  307  VRVOELPK-LIDATHPPLYRPVTWSEYLGIKSEHLTKALSLIRINHNTNPSLTGLIGNDEPKSINVDSDKTILAVFG
NtGA3ox   310  VKVSELAK-LVDORHPPLYRAVTWSEYLGTRARHFDKALSSVRLCAPLS----GFTDAKDHNGV----------OVG
StGA3ox   312  VRVSELSK-LVDHRHPPLYRAVTWSEYLGTRAKYFDKALSSVRLCVPLN----GFTDAKDHKGV----------OVG
PsGA3ox   311  VEICEHAK-LIIGPTKPPLYRSVTWNEYLGTRAKHFNKALSSVRLCTPIN----GLFDVNDSNKNSV--------OVG
AtGA3ox   305  YMISELPK-LVDPLOSPLYSLTWKONLATKATHFNOSLSIIRN---------------------------------
CmGA3ox   306  VEMSEHKR-LLNPTOPPLYRPISWTDFIRIKSNNFDGLSCIRVDVKDEK------------OVK----------VG
CmGA23ox  303  LYVAEYCKPLSETLOTPIIMKGVNVKEYFIIKAKASGKGIAALTI------------------------------
OsGA3ox   308  AEVALELPE-AVPAGRSLAYRAVTWPEYMAVRKKAFATGGSALKMVSTDAAAAADEHDDVAAAADVHA----------
```
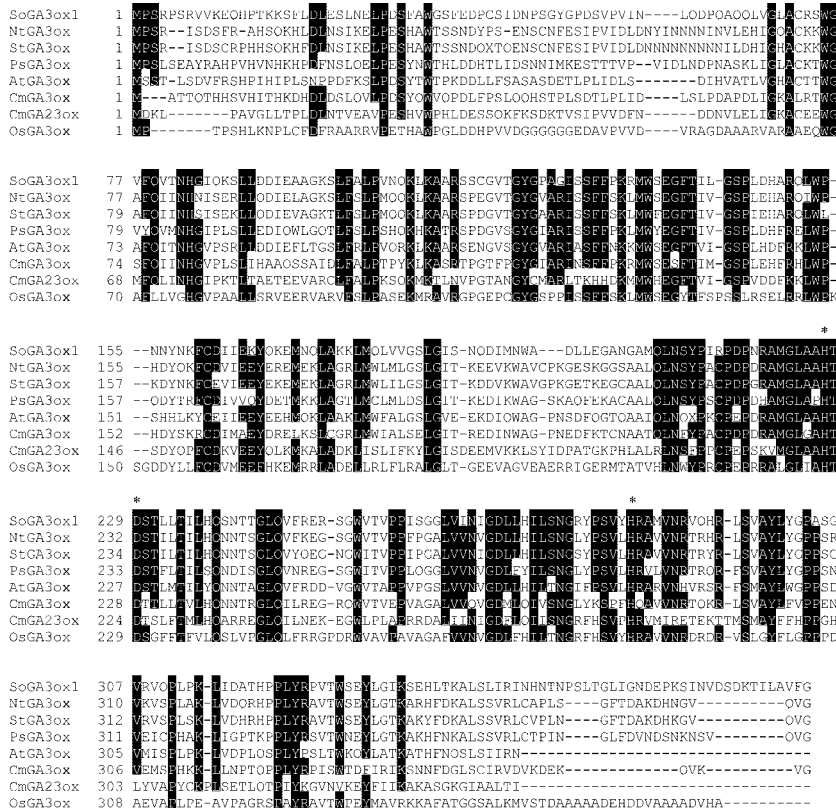
**Figure 2.** Alignment of the deduced amino acid sequences for the *SoGA3ox1* gene from spinach with other GA 3-oxidases. Conserved regions are boxed in black. Asterisks indicate the putative $Fe^{2+}$-binding motif at the active site of 2-oxoglutarate-dependent dioxygenases (Thomas et al., 1999). SoGA3ox1, GA 3-oxidase from spinach (GenBank accession no. AF506280); NtGA3ox, GA 3-oxidase from tobacco (*Nicotiana tabacum*; accession no. BAA89316); StGA3ox, GA 3-oxidase from potato (*Solanum tuberosum*; accession no. AAK91507); PsGA3ox, GA 3-oxidase from garden pea (GenBank accession no. AAC96015); AtGA3ox, GA 3-oxidase from Arabidopsis (accession no. T51691); CmGA3ox and CmGA23ox, GA 3-oxidases from pumpkin (*Cucurbita pepo*; accession nos. AAB64347 and CAB92914); and OsGA3ox, GA 3-oxidase from rice (*Oryza sativa*; accession no. BAB62072).

1999). It shares 33% to 56% identity with the GA 3-oxidases listed in Figure 2.

The primer combination JZ254 and JZ258 yielded a 236-bp fragment of GA 2-oxidase1, and JZ 254 and JZ 342 yielded a 119-bp fragment of GA 2-oxidase2 (data not shown). The sequence of each RT-PCR product was homologous to the GA 2-oxidase genes of runner bean and Arabidopsis (Thomas et al., 1999) and pea (Lester et al., 1999). 5'- and 3'-RACE were performed with sequence-specific primers (JZ282 and JZ298 for GA 2-oxidase1, and JZ375 and JZ574 for GA 2-oxidase2; Table I) using the SMART RACE cDNA amplification kit (BD Biosciences Clontech). The coding region of the *SoGA2ox1* gene was obtained with primers JZ300 and JZ301 (Table I). The full-length *SoGA2ox1* cDNA clone (1,229 bp, accession no. AF506281) has an ORF of 1,014 bp and encodes a protein of 337 amino acids with a 114-bp 5'-untranslated sequence and a 101-bp 3'-untranslated sequence. The molecular mass of the predicted protein is 38 kD, with an pI of 8.46. The SoGA2ox1 protein shares 40% to 61% identity with the other GA 2-oxidases listed in Figure 3. Northern-blot analysis showed that the size of the *SoGA2ox1* transcript is 1.3 kb (data not shown).
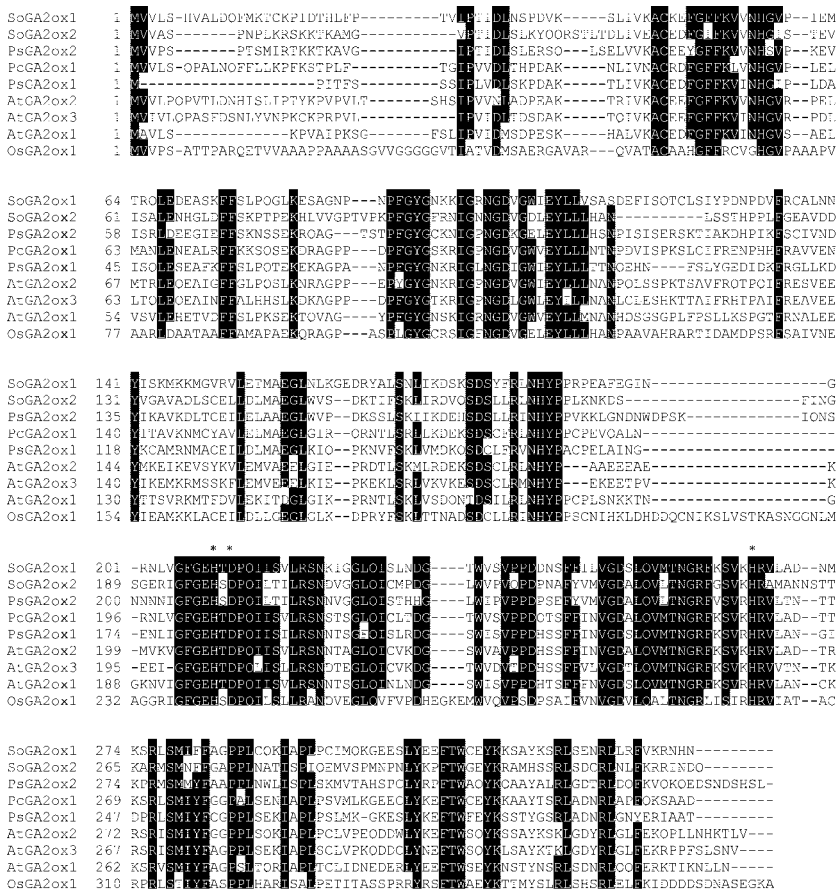
The full-length sequence of the second GA 2-oxidase (1,130 bp), designated *SoGA2ox2* (accession no. AF506282), contains an ORF of 990 bp encoding a protein of 329 amino acids (36 kD) with a 113-bp 5'-untranslated sequence, and a 27-bp 3'-untranslated sequence with an pI of 7.1. The coding region of the *SoGA2ox2* gene was obtained by PCR with the sequence-specific primers JZ619 and JZ582 (Table I). SoGA2ox2 shares 37% to 55% identity with other GA 2-oxidases listed in Figure 3 and has a low identity (40%) with the SoGA2ox1 protein. We established by dot-blot analysis that there is no cross-reactivity between the *SoGA2ox1* and *SoGA2ox2* genes (data not shown). Figure 3 shows the conserved regions among various GA 2-oxidases. Alignment with GA 2-oxidases from other species indicates that the two putative GA 2-oxidases from spinach belong to the same class of proteins and contain highly conserved amino acid sequences that may bind iron at the active site.

## Heterologous Expression in *Escherichia coli*

To determine that the *SoGA3ox1* cDNA clone encodes a GA 3-oxidase and that the *SoGA2ox1* and *SoGA2ox2* clones encode GA 2-oxidases, each coding region was heterologously expressed as a fusion protein of the glutathione *S*-transferase fusion vector in *E. coli*, strain BL21p Lys21. Soluble protein extracts were used for assays of GA 3-oxidase and GA 2-oxidase activities with several radioactive GAs as substrates. The reaction products were separated by reverse phase HPLC with an on-line radioactivity detector. Retention times (Rts) of the products were compared with those of standard ^{14}C-labeled GAs for

**Figure 3.** Alignment of the deduced amino acid sequences for the *SoGA2ox1* and *SoGA2ox2* genes from spinach with other GA 2-oxidases. Conserved regions are boxed in black. Asterisks indicate the putative $Fe^{2+}$-binding motif at the active site of 2-oxoglutarate-dependent dioxygenases (Thomas et al., 1999). SoGA2ox1 and SoGA2ox2, GA 2-oxidases from spinach (accession nos. AF506281 and AF506282); PcGA2ox1, GA 2-oxidase from runner bean (accession no. AT132438); PsGA2ox1 and PsGA2ox2, GA 2-oxidases from garden pea (accession nos. AF100954 and AF100955); AtGA2ox1, AtGA2ox2, and AtGA2ox3, GA 2-oxidases from Arabidopsis (accession nos. AJ132435, AJ132436, and AJ132437); OsGA2ox1, GA 2-oxidase from rice (accession no. BAB40934).

```
SoGA2ox1   1  MVVLS-HVALDQFMKTCKPIDTHLFP---------TVIPLIDLNSPDVK-----SLIVKACKLFGFFKVVNIGVP--IEM
SoGA2ox2   1  MVVAS-------PNPLKRSKKTKAMG-----------VPFIDLSLKYQORSTLTDLIVEACGLDICATKVWNHCLS--TEV
PsGA2ox1   1  MVVPS-------PTSMIRTKKTKAVG-----------IPFIDLSLERSO---LSELVVKACFEYGFFKVWNHSVP--KEV
PcGA2ox1   1  MVVLS-OPALNOFFLLKPFKSTPLF----------TGIPVVDLTHPDAK-----NLIVNAGRDFGFFKLVNHGVP--LEL
PsGA2ox1   1  M-------------------PITFS--------SSIPLVDLSKPDAK-----TLIVKAGEDFGFFKVNHCIP--LDA
AtGA2ox2   1  MVVI.POPVTLDNHTSLIPTYKPVPVLT-------SHSIFPVVNIADPEAK----TRIVKAGFEFGFFKVVNHGVR--PRI
AtGA2ox3   1  MTIVI.QPASFDSNLYVNPKCKPRPVL----------IPVTDLTDSDAK----TQIVKAGFEFGFFKVTNHGVR--PDL
AtGA2ox1   1  MAVLS----------KPVAIPKSG------FSLLVVIDMSDPESK-----HALVKAGEDFGFFKVWNHGVS--AEL
OsGA2ox1   1  MVVPS-ATTPARQETVVAAAPPAAAAASGVVGGGGGVVTATVEMSAERGAVAR---QVATAGAAHGFFRCVGHGVPAAAPV

SoGA2ox1  64  TRQLEDEASKFFSLPOGLKESAGNP---NPFGYGNKKIGRNGDVGWIEYLLVVSASDEFISOTCLSIYPDNPDVGRCALNN
SoGA2ox2  61  ISALENHGLDFFSKPTPEKHLVVGPTVPKPFGYGFRNIGNGLVGDLEYLLLHAN----------LSSTHPPLEGEAVDD
PsGA2ox2  58  ISRLDEEGIBFFSKNSSEKROAG---TSTPFGYGCKNIGENGDKGELEYLLLHSNPISISERSKTIAKDHPIKGSCIVND
PcGA2ox1  63  MANLENEALRFFKKSOSPKDRAGPP---DPFGYSKRIGLNGDVGWIEYLLLTTNGQEHN----FSLYGEDIDKFRGLLKD
PsGA2ox1  45  ISOLPSEAFKFFSLPOTEKEAGPA---NPFGYGNKRIGINGDIGWIEYLLLTTNGOEHN----FSLYGEDIDKFRGLLKD
AtGA2ox2  67  MTRLEOEAIGFFGLPOSLKNRAGPP---EPMGYVGNKRICGNGDVGWIEYLLLNANPOLSSPKTSAVFROTPCIFRESVEE
AtGA2ox3  63  LTOLEOEAINFFALEHSLKDKAGPP---DPFGYTKRICGNGDIGWLEYLFLNANLCLESHKTTAIFRHTPAIFREAVEE
AtGA2ox1  54  VSVLLEHETVDFFSLPKSEKTOVAG----YPFGYVGNSKIGRNGDVGWVEYLLLMNANHDSSSGPLFPSLLKSPGTFRNALEE
OsGA2ox1  77  AARLEDAATAARFRAMAPAEKQRAGP---ASPLGYGCRSIGENGDVGELEYLLTHANPAAVAHRARTIDAMDPSREGAIVNE

SoGA2ox1 141  YISKMKKMGVRVLETMAEGLNLKGEDRYALSNLIKDSKSDSYFRLNHYPPRPEAFEGIN--------------------G
SoGA2ox2 131  MVGAVADLSCELDLMAEGLWVS--DKTIFSKLIRDVOSDSLLRFANIYPPLKNKDS-------------------FING
PsGA2ox2 135  MIKAVKDLTCEIELLAAEGLWVP--DKSSLSKIIKDELKDEKSDSLLRFANIYPPVKKLGNDNWDFSK---------IONS
PcGA2ox1 140  MITAVKNMCYAVLELMAEBGLGIR--ORNTLSEKLLKDEKSDSCRRFANIYPCPEVOALN---------------------
PsGA2ox1 118  YKCAMRNMACEIDLMAEBGLKIO--PKNVFSKLVMDKOSDCLERVNHYPACPELAING----------------------
AtGA2ox2 144  YMKEIKEVSYKVLEMVAEFLGIE--PRDTLSKMLRDEKSDSCLRFANHYP----AAEEEAE------------------K
AtGA2ox3 148  YIKEMKRMSSKFLEMVEFLKIE--PKEKLSRLVKVKESDSCLRMNHYP---EKKEETPV-----------------K
AtGA2ox1 130  YTTSVRKMTFDVLEKITLGLGIK--PRNTLSKLVSDONTPSILRFNHYPPCPLSNKKTN-----------------
OsGA2ox1 154  YIEAMKKLACEIELDLLGEGIGLX--DPRYFSKETTNADSDCLLRFNHYRPSCNIHKLDHDDQCNIKSLVSTKASNGGNLM

                                                          *  *                                    *
SoGA2ox1 201  -RNLVGFGEHTDPOIISVLRSNKIGGLOISLNDG----IMVSVPPDUNSFHILVGLSLOVMTNGRMKSVKHRVLAD--NM
SoGA2ox2 189  SGERIGFGEHSDPOLETILRSNDVGGLOICMPDG----LDVFVOPDPNAFYVMVGALOVFTNGRFFSVKHRAMANNSTT
PsGA2ox1 200  NNNNIGFGEHSDPOIETTILRSNNVGGLOISTHHG----LAIPVPPDPSEFYVMVGDALOVITNGRFSVRHRVLTN--TT
PcGA2ox1 196  -RNLVGFGEHTDPOIISVLRSNTSGLOICLTDG----TMVSVPPDOTSFFINVGDALOVMTNGRFPKSVKHRVLAD--TT
PsGA2ox1 174  -ENLIGFGEHTDPOIISLLRSNNTSGLOISLRDG----SWISVPPDHSSFFINVGLSLOVMTNGRFPKSVRHRVLAN--GI
AtGA2ox2 199  -MVKVGFGEHTDPOILSVLRSNNTAGLOICVKDG----TSVAVPPDHSSFFINVGDALOVMTNGRFAKSVKHRVLAD--TR
AtGA2ox3 195  -EEI-GFGEHTDPOIESLLRSNDTEGLOICVKDG----TFDVVPDHSSFFVLVGTLOVMTNGRFAKSVKHRVVTN--TK
AtGA2ox1 188  GKNVIGFGEHTDPOIISVLRSNNTSGLOINLNDG----SWISVPPDHTSFFFNVGLSLOVMTNGRFPKSVRHRVLAN--CK
OsGA2ox1 232  AGGRIGFGEHSDPOIISLLRANDVEGLOVFVPDHEGKEMMVQVESDPSAIRVNVGRVLOATTNGRLISIRHRVMIAT--AC

SoGA2ox1 274  KSRLSMTFFAGPPLCOKIAPLPCIMOKGEESLYEEFTWCEYKKSAYKSRLSENRLRFVKRNHN---------
SoGA2ox2 265  KARMSMNFFGAPPLNATHSPLOEMVSPMNPNLYKPFTWGEYTRAMHSSRLSDGRINLFKRRINDO--------
PsGA2ox1 274  KDRMSMMYFAAPDLNWLHSPDISKMVTAHSPCLYRFPFTWAGYKCAAYRALRGDTRLCOFKVOKOEDSNDSHSL-
PcGA2ox1 269  KSRLSMIYFGAPDLSEKHAPLPSVMLKGEECLYKEFTWCEYKKAAYISRLADNRLADFKOKSAAD---------
PsGA2ox1 247  DBRLSMIYFCGPPLSEKIAPLPSLMK-GKESLYKEETWFEYKSSTYGSRLADNRLGNYERIAAT-----
AtGA2ox2 272  RSRISMIYFAGPPLSOKHAPLPCLVPEODDWYKEFTWSGYKSSAYKSKLGDYRLGLFEKOPLLNHKTLV---
AtGA2ox3 267  RSRISMIYFAGPPLSEKHAPLSCLVPKODDCLYNEFTWSOYKLSAYKTKLGDYRLGLFEKRPPFSLSNV----
AtGA2ox1 262  KSRVSMIYFAGPSLTORHAPTCLIDNEDERLYEEFTWSEYKNSTYNSRLSDNRLCOFERKTIKNLLN-----
OsGA2ox1 310  RPRLSITIYFASPPLHARHSALPETITASSPRRMRSFTWAEYKRTMYSLRHSHSRFLFLFKIDDDDSDNASEGKA
```

tentative identification. The SoGA3ox1 protein converted radioactive $GA_9$ and $GA_{20}$ to products with Rts of 27.8 and 15.4 min, corresponding to those of $GA_4$ and $GA_1$, respectively. The product of $[^{14}C_4]GA_{20}$ (specific activity 20 $\mu$Ci $\mu mol^{-1}$) incubated with SoGA3ox1 was identified as $[^{14}C_4]GA_1$ (Table II), thus demonstrating conclusively that *SoGA3ox1* encodes a GA 3-oxidase.

Both SoGA2ox recombinant proteins converted radioactive $GA_9$ and $GA_{20}$ to putative $GA_{51}$ (Rt = 25.8 min) and $GA_{29}$ (Rt = 10.1 min), respectively. The products of $[^{14}C_4]GA_{20}$ and $[^2H_2]GA_{20}$ metabolism were identified as $[^{14}C_4]GA_{29}$ and $[^2H_2]GA_{29}$, respectively; $[^2H_2]GA_1$ was converted to $[^2H_2]GA_8$ (Table II), so that both $GA_{20}$ and $GA_1$ are substrates for these GA 2-oxidases. In addition to $GA_{29}$, a second product (Rt = 15.0 min) was formed in incubations of $GA_{20}$ with SoGA2ox1. This product was tentatively identified as $GA_{29}$-catabolite, because $[^{14}C_4]GA_{29}$ was converted by SoGA2ox1 to a product with the same Rt.

SoGA2ox1, but not SoGA2ox2, also converted $GA_{53}$ (Rt = 29.3 min) to $GA_{97}$ (Rt = 12.4 min; Table II) and a second metabolite (Rt = 17.0 min), which was not identified. Thus, it appears from these results that the substrate specificity of the two GA 2-oxidases isolated from spinach is similar to that of other GA 2-oxidases (Thomas et al., 1999; Elliott et al., 2001) in that they all catalyze 2$\beta$-hydroxylation of $C_{19}$-GAs,

but that SoGA2ox1 is also capable of 2$\beta$-hydroxylation of the $C_{20}$-GA, $GA_{53}$.

## Gene Expression in SD and LD Conditions

*SoGA2ox1* transcripts were barely detectable on northern blots, so *SoGA2ox1* gene expression was measured by the quantitative RT-PCR method (Cowling et al., 1998). For the PCR reaction, 29 cycles were chosen as the point where PCR products of the *SoGA2ox1* and *SoActin* genes were increasing exponentially (data not shown).

To investigate how the photoperiod regulates expression of the GA dioxygenases isolated, spinach plants grown in SD conditions were transferred to LD conditions for 8 d. Figure 4 shows the expression patterns of four GA dioxygenases in several organs. In SD conditions, the levels of the *SoGA20ox1* transcripts were low in blades, petioles, and young leaves, but the *SoGA20ox1* transcripts accumulated in all organs tested, especially in petioles and tips, when spinach plants were transferred from SD to LD conditions.

The levels of the *SoGA3ox1* transcripts of plants in SD conditions were lower in petioles and tips than in leaf blades and young leaves. There was a slight increase in *SoGA3ox1* transcript levels in leaf blades and petioles when spinach plants were transferred

**Table II.** *Identification of products after incubation of recombinant GA 2-oxidases (SoGA2ox1 and SoGA2ox2) and GA 3-oxidase (SoGA3ox1) from spinach with different substrates*

| Enzyme | Substrate | Product | Mass Spectra of Products[a] |
|--------|-----------|---------|------------------------------|
| | | | *m/z (% relative abundance)* |
| SoGA2ox1 | $[^{14}C_4]GA_{20}$ | $[^{14}C_4]GA_{29}$ | $M^+$ 514 (7), 506 (100), 491 (12), 477 (4), 447 (6), 389 (7), 375 (9), 303 (14), 277 (3), 235 (5), 207 (17), 167 (5) |
| SoGA2ox1 | $[^2H_2]GA_1$ | $[^2H_2]GA_8$ | $M^+$ 596 (100), 581 (7), 537 (4), 506 (2), 450 (10), 381 (6), 313 (2), 283 (3), 240 (7), 209 (10) |
| SoGA2ox1 | $[^2H_2]GA_{53}$ | $[^2H_2]GA_{97}$ | $M^+$ 538 (35), 523 (11), 506 (11), 479 (14), 448 (10), 417 (8), 388 (11), 373 (9), 357 (6), 329 (21), 289 (4), 259 (5), 239 (47), 209 (100), 179 (15) |
| SoGA2ox2 | $[^2H_2]GA_{20}$ | $[^2H_2]GA_{29}$ | $M^+$ 508 (100), 493 (11), 479 (4), 449 (6), 391 (8), 359 (4), 305 (18), 279 (3), 237 (5), 209 (23), 169 (6) |
| SoGA2ox2 | $[^2H_2]GA_1$ | $[^2H_2]GA_8$ | $M^+$ 596 (100), 581 (12), 537 (10), 506 (9), 450 (16), 381 (14), 313 (4), 283 (7), 240 (12), 209 (16) |
| SoGA3ox1 | $[^{14}C_4]GA_{20}$ | $[^{14}C_4]GA_1$ | $M^+$ 514 (7), 506 (100), 491 (12), 448 (16), 16 (3), 376 (13), 357 (3), 313 (7), 235 (6), 207 (20), 193 (8), 180 (5) |

[a] As the methyl ester trimethylsilyl ethers.

from SD to LD conditions, but the change in photoperiod had very little effect on transcript level in young leaves and tips (Fig. 4), the organs where growth takes place.

The levels of the *SoGA2ox1* transcripts in SD-grown plants were lower in leaf blades than in petioles, young leaves, and tips. But in LD conditions, the
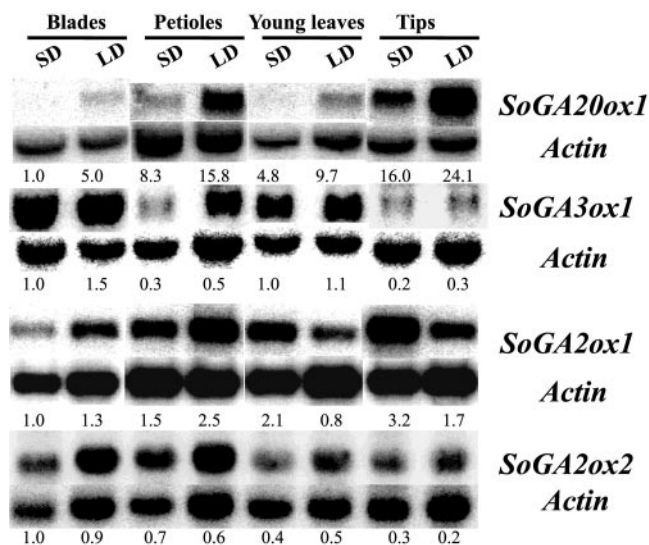


**Figure 4.** Expression patterns of the *SoGA20ox1*, *SoGA3ox1*, *SoGA2ox1*, and *SoGA2ox2* genes in various organs of spinach plants grown in SD and after 8 LD. Northern blots were prepared by separating 30 μg of total RNA of each organ through 1.2% (w/v) agarose gels containing formaldehyde, followed by transfer to Hybond-N⁺ membranes. The blots were separately hybridized to ³²P-labeled cDNAs of *SoGA20ox1*, *SoGA3ox1*, *SoGA2ox2*, or *SoActin*. After RT-PCR of *SoGA2ox1* and *SoActin*, 10 μL of the PCR reaction products were separated through 2.0% (w/v) agarose gel by electrophoresis and hybridized to a ³²P-labeled cDNA of *SoGA2ox1* or *SoActin*. The numbers under the blots indicate the relative amount of each transcript after standardization using *SoActin* as a loading control. The value for each gene transcript in blades of spinach grown in SD conditions was arbitrarily set at 1.0.

levels of the *SoGA2ox1* transcripts were lower in young leaves than in other organs. Thus, the levels of the *SoGA2ox1* transcripts increased in blades and petioles when spinach plants were transferred from SD to LD conditions. By contrast, the level of *SoGA2ox1* transcripts decreased in young leaves and tips. This decrease in the level of *SoGA2ox1* transcripts in young leaves and tips in LD conditions is opposite to the increase in the level of *SoGA20ox1* transcripts.

The *SoGA2ox2* gene was highly expressed in leaf blades and petioles. However, there were no significant differences between transcript levels in SD and LD, so that expression of *SoGA2ox2* was not further investigated in subsequent experiments.

### Time Course of LD Treatment

Spinach plants were harvested at different time intervals after transfer from SD to LD conditions to determine the time course of changes in gene expression. The levels of the *SoGA20ox1*, *SoGA3ox1*, and *SoGA2ox1* transcripts were all up-regulated in petioles in a time-dependent manner (Fig. 5A). This enhancement was much stronger in the case of the *SoGA20ox1* gene (26×) than of the *SoGA3ox1* gene (1.6×) and of the *SoGA2ox1* gene (4×). The levels of the *SoGA20ox1* transcripts were also increased time-dependently in young leaves and tips (Fig. 5, B and C). LD conditions caused more rapid accumulation of the *SoGA20ox1* transcripts in tips than in petioles and young leaves. However, the *SoGA3ox1* mRNA abundance in young leaves and tips was similar or only slightly increased by the LD treatment (Fig. 5, B and C). By contrast, the levels of the *SoGA2ox1* transcripts were down-regulated in young leaves and tips (Fig. 5, B and C). The levels of the *SoGA2ox1* transcripts were more rapidly decreased in young leaves than in tips. This rapid decrease may compensate for the relatively low increase in the levels of the *SoGA20ox1*
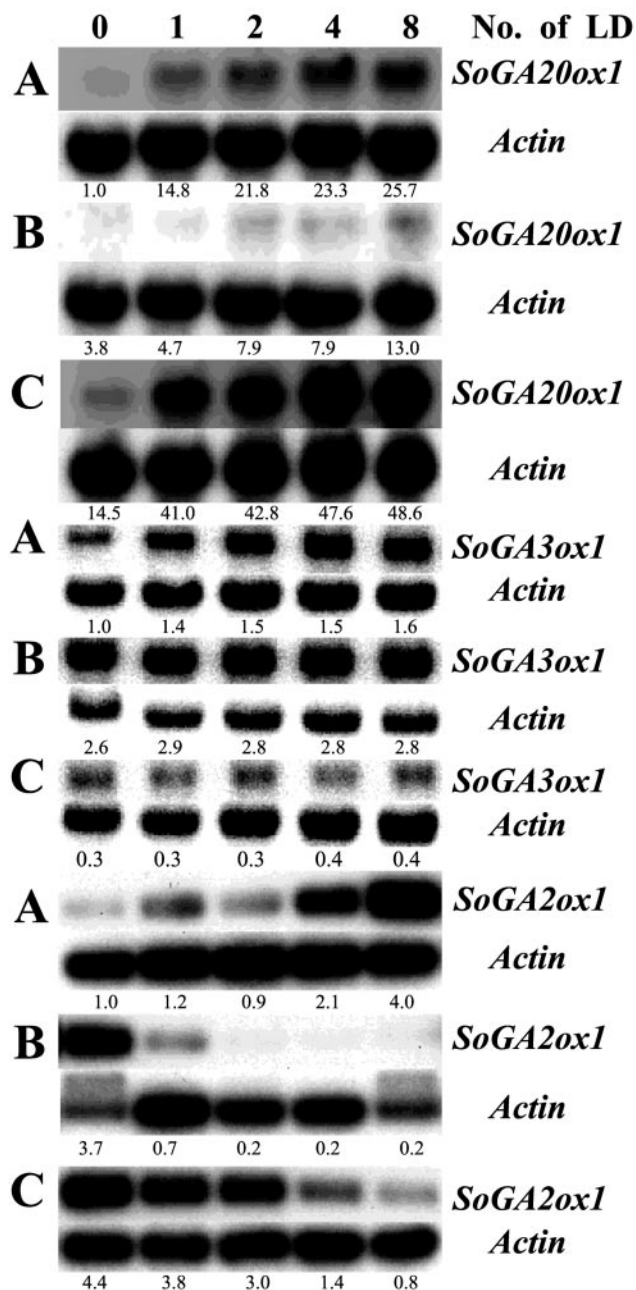
**Figure 5.** Time course of *SoGA20ox1*, *SoGA3ox1*, and *SoGA2ox1* expression after spinach plants were transferred from SD to LD conditions. *GA20ox1* transcripts increased with increasing duration of LD treatment in petioles (A), young leaves (B), and tips (C). *GA3ox1* transcripts increased with increasing duration of LD treatment in petioles (A), but they did not change in young leaves (B), and tips (C) after exposure to increasing numbers of LD. *GA2ox1* transcripts increased with increasing duration of LD treatment in petioles (A), but they decreased in young leaves (B), and tips (C). The numbers under the blots indicate the relative amount of transcript after standardization using *SoActin* as a loading control. The value of each transcript level in petioles in SD was arbitrarily set at 1.0.

and *SoGA3ox1* transcripts in young leaves to maintain the active GAs. The time course experiment demonstrates the different regulation by the photo-

period of the three GA dioxygenases: (a) expression of *SoGA20ox1* changed quickly and strongly in petioles and tips, (b) the *SoGA3ox1* transcript level was only slightly changed in petioles, and (c) the *SoGA2ox1* transcript level increased in petioles but decreased in young leaves and tips.

## Effect of Light and Darkness on Gene Expression

To investigate the effect of light and darkness on gene expression, spinach plants grown in SD and LD conditions were harvested at the end of the 8-h high-intensity light period (0 h), at the end of 16 h of darkness (16 D), or at the end of 16 h of low-intensity light (16 L). The levels of the *SoGA20ox1*, *SoGA3ox1*, and *SoGA2ox1* transcripts were determined in petioles, young leaves, and tips (Fig. 6). Compared with the levels in each group at the end of the 8-h high-intensity light period (0 h), additional light (16 L) greatly increased the levels of the *SoGA20ox1* transcripts in petioles of plants that were initially in SD conditions ($13\times$ compared with SD at 0 h) and also in petioles in LD conditions ($4\times$ compared with LD at 0 h; Fig. 6A). The levels of the *SoGA20ox1* transcripts in tips were not changed by additional light (Fig. 6C). A long dark treatment (16 D) did not affect the levels of the *SoGA20ox1* transcripts in petioles, young leaves, and tips in either SD or LD conditions. This means that the levels of *SoGA20ox1* transcript are regulated much more by light than by darkness. Exposure to weak light or dark treatment had little effect on the levels of the *SoGA3ox1* and *SoGA2ox1* transcripts (Fig. 6).

## Expression in Other Organs

To determine other organ-specific expression patterns of the GA dioxygenases, male and female flowers, stems, and shoot tips were harvested from spinach plants that had been exposed to LD conditions for more than 3 weeks (Fig. 7). The shoot tips included the upper 1 cm; stems were the next 1 cm of the elongated stem. The *SoGA20ox1* mRNA was more abundant in stems than in shoot tips, and little transcript was detected in flowers. By contrast, *SoGA3ox1* and *SoGA2ox1* were highly expressed in male and female flowers, stems, and shoot tips.

## DISCUSSION

We isolated a full-length cDNA clone encoding GA 3-oxidase and two full-length cDNA clones encoding GA 2-oxidase from spinach. Heterologous expression in *E. coli* showed that the product of the *SoGA3ox1* gene is able to catalyze 3β-hydroxylation of $GA_{20}$ to $GA_1$, whereas the products of the *SoGA2ox* genes catalyze 2β-hydroxylation of $GA_{20}$ and $GA_1$, both $C_{19}$-GAs with a γ-lactone in the A ring of the GA structure (Fig. 1). In addition, recombinant
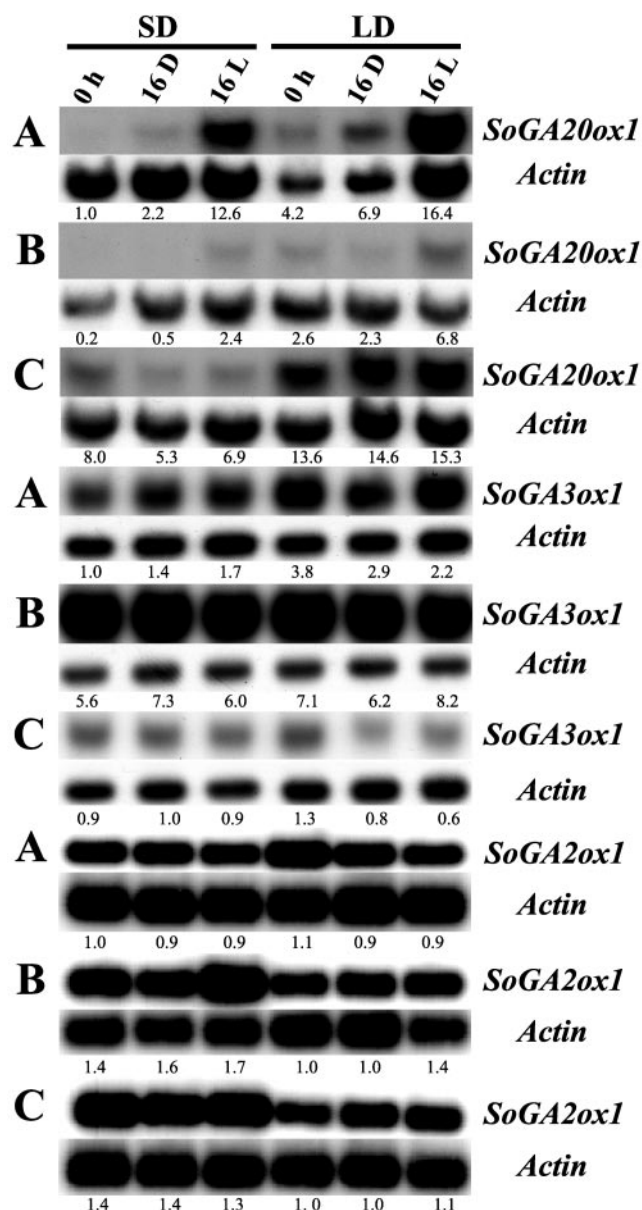
**Figure 6.** Effects of light and darkness on the transcript level of *SoGA20ox1*, *SoGA3ox1*, and *SoGA2ox1* in petioles (A), young leaves (B), and tips (C). Spinach plants were harvested at the end of the 8-h high-intensity light period (0 h) and after transfer from SD or LD conditions to 16 h of darkness (16 D) or to 16 h of weak incandescent light (16 L). The numbers under the blots indicate the relative amount of each transcript after standardization using *SoActin* as a loading control. The value of each transcript level in petioles at 0 h in SD was arbitrarily set at 1.0.



**Figure 7.** Expression of *SoGA20ox1*, *SoGA3ox1*, and *SoGA2ox1* in male flowers (♂), female flowers (♀), stems (St), and shoot tips (ShT). Spinach plants were harvested after more than 3 weeks in LD conditions. In this case, the shoot tips included the upper 1 cm of the shoots; stems were the next 1 cm of the elongated stems. The numbers under the blots indicate the relative amount of each transcript after standardization using *SoActin* as a loading control. The value of the transcript level of each gene in male flowers was arbitrarily set at 1.0.

SoGA2ox1 converts $GA_{53}$ to $GA_{97}$, both $C_{20}$-GAs (Fig. 1). The recombinant proteins of GA 2-oxidases from other species specifically 2β-hydroxylate $C_{19}$-GAs (Thomas et al., 1999; Elliott et al., 2001). So far, few exceptions have been reported to this rule: $GA_{15}$, a $C_{20}$-GA, was 2β-hydroxylated by PcGA2ox1 and AtGA2ox2 (Thomas et al., 1999); and $GA_{44}$, also a $C_{20}$-GA (Fig. 1), was 2β-hydroxylated to $GA_{98}$ by

OsGA2ox1 (Sakamoto et al., 2001). Both of these GAs have a δ-lactone in the A ring. When this lactone ring was opened, $GA_{15}$ was no longer metabolized by recombinant PcGA2ox1, but it was (although less efficiently) by AtGA2ox2 (Thomas et al., 1999). Spinach produces large amounts of 2β-hydroxy-$GA_{53}$ (=$GA_{97}$), and smaller amounts of $GA_{98}$ (= 2β-hydroxy-$GA_{44}$), $GA_{99}$ (= 2β-hydroxy-$GA_{19}$; Mander et al., 1996), and $GA_{110}$ (= 2β-hydroxy-$GA_{12}$; Owen et al., 1998). Substrate specificity of the 2-oxidases for the possible precursors of these 2β-hydroxy-$C_{20}$-GAs remains to be determined.

Expression of *SoGA3ox1* was very little affected by the photoperiod (Figs. 4 and 5), so that it is possible that the conversion of $GA_{20}$ to $GA_1$ in LD is limited by the amount of $GA_{20}$ substrate available and not by the enzyme level. Although expression of *SoGA2ox1* was down-regulated by LD, this may be only a minor factor in the maintenance of a high $GA_1$ level, because *SoGA2ox1* transcripts are much less abundant than those of *SoGA2ox2*, and expression of the latter is not under photoperiodic control (Fig. 4). In a different system, de-etiolation of pea seedlings, GA 3-oxidase was down-regulated by light, whereas GA 2-oxidase was up-regulated. This resulted in decreased $GA_1$, increased $GA_8$ levels, and a reduction in growth rate (Reid et al., 2002).

The increase in growth rate of petioles of spinach in LD occurs during the first 16 h of weak supplementary light (Zeevaart, 1971). This may be mainly because of the increase in *SoGA20ox1* transcripts during

the additional 16 h of light rather than the small increase in *SoGA3ox1* and decrease in *SoGA2ox1* transcripts (Fig. 6A). The results in Figure 6 show that moving spinach plants from LD to 16 h darkness did not reduce the transcript level of any of the GA dioxygenases studied. On the other hand, Gilmour et al. (1986) reported that enzyme activities for the conversions $GA_{53}$ to $GA_{44}$ and $GA_{19}$ to $GA_{20}$, both representing GA 20-oxidase activity, declined quickly in plants in darkness. It is possible, therefore, that regulation of GA 20-oxidase by photoperiod is not only at the transcriptional level, but that there may be posttranscriptional control as well. Thus, transcript levels may not necessarily be indicative of enzyme activity. Further work on photoperiodic control of GA biosynthesis in spinach should therefore attempt to compare the protein level and enzymatic activity of GA 20-oxidase.

The low expression of the *SoGA20ox1* gene in spinach flowers (Fig. 7) may mean that it has no role in flower development, unlike the *LeGA20ox1* and *LeGA20ox2* genes, which are highly expressed in tomato (*Lycopersicon esculentum*) during flower bud and early fruit development (Rebers et al., 1999). Thus, another GA 20-oxidase gene may be involved in flower development in spinach.

Up-regulation of GA biosynthesis by LD starts at the beginning of the pathway with increased *ent*-kaurene biosynthesis (Zeevaart and Gage, 1993), and in the later stages, it is mainly attributable to the much increased expression of *SoGA20ox1*. It is surprising that expression of *SoGA3ox1*, encoding the product that catalyzes the pivotal step of 3$\beta$-hydroxylation, shows little fluctuation in response to the photoperiod (Fig. 4). Although expression of *SoGA2ox1* is down-regulated in tips and young leaves of plants in LD, the overall effect of LD on GA metabolism is that more $GA_{29}$ (Metzger and Zeevaart, 1980, 1982) and $GA_8$ (Wu et al., 1996) are produced in plants in LD than in SD. Thus, it appears that in SD, the steps catalyzed by GA 20-oxidase are enzyme limited, whereas GA 3-oxidase and GA 2-oxidase are limited by the amount of substrate available. In LD, a new balance is established between $GA_1$ synthesis and deactivation, increasing the $GA_1$ level to such an extent that the threshold value required for stem elongation is exceeded (Zeevaart et al., 1993).

## MATERIALS AND METHODS

### Plant Material and Growing Conditions

Spinach (*Spinacia oleracea* L. cv Savoy Hybrid 612) plants were grown in a SD growth chamber for approximately 6 or 7 weeks as described previously (Zeevaart and Gage, 1993). SD conditions consisted of 8 h of light from fluorescent tubes and incandescent bulbs of approximately 300 $\mu$mol m$^{-2}$ s$^{-1}$ at 23°C, followed by 16 h darkness at 20°C. For LD conditions, the 8-h main light period was followed by 16 h of weak light from incandescent bulbs of 10 $\mu$mol m$^{-2}$ s$^{-1}$ at 20°C.

### RT-PCR

RT-PCR was used for the cloning of GA 3-oxidase and GA 2-oxidases. First-strand cDNA from total RNA was synthesized by SuperScript II RNase H-minus Reverse Transcriptase (Invitrogen, Carlsbad, CA), using oligo(dT) primers at 42°C for 1 h. The cDNA product was then used in PCR reactions containing *Taq* polymerase and several pairs of degenerate primers (Table I). For GA 3-oxidase, degenerate primers were synthesized based on conserved regions of GA 3-oxidases from Arabidopsis (Chiang et al., 1995; Yamaguchi et al., 1998) and pea (Lester et al., 1997). For GA 2-oxidase, the degenerate primers were synthesized based on conserved regions of GA 2-oxidases from runner bean and Arabidopsis (Thomas et al., 1999). These primers were used in all combinations in PCR reactions with first-strand cDNA as a template. The PCR amplifications were performed with a DNA Thermal Cycler (RTC-200, MJ Research, Waltham, MA) in a 50-$\mu$L reaction containing 1× PCR buffer (Invitrogen), 1.5 mM MgCl$_2$, 200 $\mu$M dNTP, 2.5 pmol of each primer, 1 unit of *Taq* DNA polymerase (Invitrogen). The reaction mixtures were heated to 94°C for 3 min and then subjected to 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 45 s. A final extension was performed at 72°C for 10 min. A 5-$\mu$L volume of the PCR reaction products was analyzed by 1.2% (w/v) agarose gel electrophoresis, purified with a Wizard PCR purification system (Promega, Madison, WI), and then cloned into the pGEM Teasy vector (Promega). Analysis of the DNA sequences was carried out using the DNASTAR program (DNASTAR, Inc., Madison, WI). Multiple sequence alignments were performed using the Clustal W 1.8 Multiple Sequence Alignment program and printed using BOXSHADE 3.21 (http://www.ch.embnet.org/software/Box_form.html).

### RACE and Cloning

5'- and 3'-RACE of spinach GA 3-oxidase cDNA and GA 2-oxidase cDNA were performed using the SMART RACE cDNA Amplification Kit from BD Biosciences Clontech. Poly(A$^+$) RNA was isolated from spinach using the PolyATtract mRNA Isolation System IV (Promega) according to the manufacturer's instructions. After RT with primers supplied by BD Biosciences Clontech, the first-strand cDNA was used directly in 5'- and 3'-RACE PCR reactions. Primary PCR amplification reactions were achieved using a high-fidelity enzyme (*Pfu* Turbo polymerase; Stratagene, La Jolla, CA) and gene-specific primers JZ282 and JZ298 for GA 2-oxidase1, JZ375 and JZ574 for GA 2-oxidase2, and JZ445 and JZ444 for GA 3-oxidase1 to generate the 5'- and 3'-cDNA fragments, respectively. The PCR reaction consisted of the first denaturation for 3 min at 94°C, a series of 30 cycles (1 min at 94°C, 1 min at 54°C or 55°C, and 1 min at 72°C) with a final extension for 5 min at 72°C using a thermal cycler (RTC-200, MJ Research). A 5-$\mu$L aliquot of the RT-PCR and RACE reaction solution was analyzed by 1.2% (w/v) agarose gel electrophoresis. PCR products were purified and cloned into the pCR-Script Cam SK(+) cloning vector from Stratagene. These constructs were sequenced.

### Northern-Blot Analysis

Leaf blades, petioles, young leaves, tips (Zeevaart and Gage, 1993), and other organs were harvested, frozen immediately in liquid N$_2$, and stored at −80°C. Northern blots were prepared by electrophoresis of 30 $\mu$g of total RNA in the presence of formaldehyde (Sambrook et al., 1989), and RNA was transferred to nitrocellulose. Full-length cDNAs were labeled with [$^{32}$P]dCTP by the Random Primers DNA Labeling System (Invitrogen) and the radioactive probes were used to probe northern blots. Hybridization was carried out at 42°C using a 50% (v/v) formamide system (Sambrook et al., 1989). Membranes were washed twice for 10 min in 2× SSC at room temperature and then twice for 10 min in 0.2× SSC with 0.1% (w/v) SDS at 65°C (high stringency). At low stringency, the membrane was washed once for 10 min in 2× SSC at room temperature and then washed again for 10 min in 2× SSC at 65°C.

The relative amounts of mRNA were determined with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each blot was first probed with one of the various cDNAs, and then stripped and reprobed with *SoActin*. The ratio of the GA dioxygenase mRNA to *Actin* mRNA was used to compare the mRNA levels in various organs and treatments. Each experiment was repeated at least three times with similar results.

## Quantitative RT-PCR

The first-strand cDNA synthesis reactions were carried out using 5 $\mu$g of each total RNA sample, random hexamer primer, and avian RNase H-minus reverse Transcriptase. A pair of primers (JZ385 and JZ386) was used to amplify a 401-bp fragment of GA 2-oxidase1. JZ389 and JZ390 primers (Table I) were used to amplify a 324-bp fragment of the *SoActin* gene as a control. Both of these fragments can be distinguished from any contaminant of genomic DNA by size. The RT-PCR products were cloned and sequenced to verify the sequences of the fragments of the *SoGA2ox1* and *SoActin* genes. The cDNA solutions (2.5 $\mu$L) were used as a template in a standard 50-$\mu$L PCR reaction. Expression of *SoGA2ox1* and *SoActin* was compared at 29 cycles where both PCR reactions were progressing exponentially. Ten microliters of PCR reaction products was separated by electrophoresis, blotted, and hybridized.

## Expression of Recombinant GA 3-Oxidase and GA 2-Oxidase Proteins

Coding regions of 2-oxidases were produced by PCR with the following primer set designed from the RACE product: JZ300 and JZ301 for mature GA 2-oxidase1 (*Psp*AI and *Xho*I sites inserted to facilitate cloning) and JZ582 and JZ619 (*Bam*HI and *Xho*I sites inserted to facilitate cloning) for mature GA 2-oxidase2. For GA 3-oxidase, JZ509 and JZ510 (*Psp*AI and *Sal*I sites inserted to facilitate cloning) were used to get the coding region. The resulting PCR fragments were cloned into a pCR-Script Cam SK(+) cloning vector and then digested with *Psp*A1/*Xho*I, *Bam*HI/*Xho*I, or *Psp*A1/*Sal*I, respectively, and subcloned into the corresponding restriction sites of the pGEX-5X-2 vector (Amersham Biosciences, Piscataway, NJ). These full-length cDNA clones (pGEXGA3ox1, pGEXGA2ox1, or pGEXGA2ox2) were transformed into *Escherichia coli*, strain BL21pLysS. Fifty milliliters of freshly cultured cells was added to 1 L of Luria-Bertani medium with 100 mg L$^{-1}$ ampicillin and incubated at 37°C with vigorous shaking. When the optical density at 600 nm reached 0.6, isopropyl-$\beta$-D-thiogalactopyranoside was added to give a final concentration of 3 mM, and the culture was incubated for another 2 h. The cells were harvested and suspended in lysis buffer (100 mM Tris-HCl, pH 7.5, and 10 mg mL$^{-1}$ lysozyme), and incubated at room temperature for 10 min. Crude cell extracts were obtained by using a Sonifier Cell Disruptor 200 (Branson Ultrasonics, Danbury, CT) carried out for 10 cycles (10 × 10 s) on ice. The lysates were submerged in liquid N$_2$ for 2 min and then thawed in an ice bath for 15 min (Johnson and Hecht, 1994). The lysates were centrifuged at 13,000 rpm for 30 min, and the supernatant was stored at −80°C until used for enzyme assays.

## Enzyme Assays and Product Identification

The assays for GA 3-oxidase activity and GA 2-oxidase activities were performed with approximately 30,000 dpm of $^{14}$C-labeled GAs. [$^2$H$_2$]GAs were used only for product identification. The reaction mixture (100 $\mu$L) contained 100 mM Tris buffer (pH 7.5), 0.5 mM FeSO$_4$, 5 mM 2-oxoglutarate, 5 mM ascorbate, and 80 $\mu$L of bacterial extract. Cofactors were added again after 1 h. The mixture was incubated at 30°C for up to 6 h with gentle shaking. For identification of products, the assays were scaled up to 500 $\mu$L. The products were isolated by reverse phase HPLC with an on-line FLO-ONE detector (Radioanalytic, Tampa, FL; Zeevaart and Gage, 1993). For identification of the products by gas chromatography (GC)-mass spectrometry (Zeevaart et al., 1993), the GC was equipped with a DB-5MS capillary column (30-m × 0.32-mm × 0.25-$\mu$m film, J&W Scientific, Folsom, CA), which was operated in splitless mode. The oven temperature was kept at 100°C for 1 min after sample injection and then programmed from 100°C to 230°C at 40°C min$^{-1}$, from 230 to 280°C at 8°C min$^{-1}$, and finally to 300°C at 20°C min$^{-1}$. Specific activities of substrates and products were determined by GC-selected ion monitoring of the molecular ion clusters (Zeevaart and Gage, 1993).

## Distribution of Materials

Upon written request, all novel materials described in this publication will be made available in limited quantities and in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

## LITERATURE CITED

**Chiang H-H, Hwang I, Goodman HM** (1995) Isolation of the Arabidopsis *GA4* locus. Plant Cell **7:** 195–201

**Cowling RJ, Kamiya Y, Seto H, Harberd NP** (1998) Gibberellin dose-response regulation of *GA4* gene transcript levels in Arabidopsis. Plant Physiol **117:** 1195–1203

**Elliott RC, Ross JJ, Smith JJ, Lester DR, Reid JB** (2001) Feed-forward regulation of gibberellin deactivation in pea. J Plant Growth Regul **20:** 87–94

**Gilmour SJ, Zeevaart JAD, Schwenen L, Graebe JE** (1986) Gibberellin metabolism in cell-free extracts from spinach leaves in relation to photoperiod. Plant Physiol **82:** 190–195

**Hedden P, Phillips AL** (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci **5:** 523–530

**Itoh H, Tanaka-Ueguchi M, Kawaide H, Chen X, Kamiya Y, Matsuoka M** (1999) The gene encoding tobacco gibberellin 3-oxidase is expressed at the site of GA action during stem elongation and flower organ development. Plant J **20:** 15–24

**Itoh H, Tanaka-Ueguchi M, Sentoku N, Kitano H, Matsuoka M, Kobayashi M** (2001) Cloning and functional analysis of two gibberellin 3$\beta$-oxidase genes that are differently expressed during the growth of rice. Proc Natl Acad Sci USA **98:** 8909–8914

**Johnson BH, Hecht MH** (1994) Recombinant proteins can be isolated from *Escherichia coli* cells by repeated cycles of freezing and thawing. BioTechnology **12:** 1357–1360

**Lester DR, Ross JJ, Davies PJ, Reid JB** (1997) Mendel's stem length gene (*Le*) encodes a gibberellin 3-oxidase. Plant Cell **9:** 1435–1443

**Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB** (1999) Gibberellin 2-oxidation and the *SLN* gene of *Pisum sativum*. Plant J **19:** 65–73

**Mander LN, Owen DJ, Croker SJ, Gaskin P, Hedden P, Lewis MJ, Talon M, Gage DA, Zeevaart JAD, Brenner ML et al.** (1996) Identification of three C$_{20}$-gibberellins: GA$_{97}$ (2$\beta$-hydroxy-GA$_{53}$), GA$_{98}$ (2$\beta$-hydroxy-GA$_{44}$) and GA$_{99}$ (2$\beta$-hydroxy-GA$_{19}$). Phytochemistry **43:** 23–28

**Metzger JD, Zeevaart JAD** (1980) Effect of photoperiod on the levels of endogenous gibberellins in spinach as measured by combined gas chromatography-selected ion current monitoring. Plant Physiol **66:** 844–846

**Metzger JD, Zeevaart JAD** (1982) Photoperiodic control of gibberellin metabolism in spinach. Plant Physiol **69:** 287–291

**Owen DJ, Mander LN, Storey JMD, Huntley RP, Gaskin P, Lenton JR, Gage DA, Zeevaart JAD** (1998) Synthesis and confirmation of structure for a new gibberellin, 2$\beta$-hydroxy-GA$_{12}$ (GA$_{110}$), from spinach and oil palm. Phytochemistry **47:** 331–337

**Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang Y-Y, Imai R, Sekimoto H, Kamiya Y** (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J **17:** 241–250

**Reid JB, Botwright NA, Smith JJ, O'Neill DP, Kerckhoffs LHJ** (2002) Control of gibberellin levels and gene expression during de-etiolation in pea. Plant Physiol **128:** 734–741

**Sakamoto T, Kobayashi M, Itoh H, Tagiri A, Kayano T, Tanaka H, Iwahori S, Matsuoka M** (2001) Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol **125:** 1508–1516

**Sambrook J, Fritch EF, Maniatis T** (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Talon M, Zeevaart JAD** (1990) Gibberellins and stem growth as related to photoperiod in *Silene armeria* L. Plant Physiol **92:** 1094–1100

**Talon M, Zeevaart JAD, Gage DA** (1991) Identification of gibberellins in spinach and effects of light and darkness on their levels. Plant Physiol **97:** 1521–1526

**Thomas SG, Philips AL, Hedden P** (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA **96:** 4698–4703

**Wu K, Gage DA, Zeevaart JAD** (1996) Molecular cloning and photoperiod-regulated expression of gibberellin 20-oxidase from the long-day plant spinach. Plant Physiol **110:** 547–554

**Yamaguchi S, Kamiya Y** (2000) Gibberellin biosynthesis: its regulation by endogenous and environmental signals. Plant Cell Physiol **41:** 251–257

**Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun T** (1998) Phytochrome regulation and differential expression of gibberellin 3$\beta$-hydroxylase genes in germinating Arabidopsis seeds. Plant Cell **10:** 2115–2126

**Zeevaart JAD** (1971) Effects of photoperiod on growth rate and endogenous gibberellins in the long-day rosette plant spinach. Plant Physiol **47:** 821–827

**Zeevaart JAD, Gage DA** (1993) *ent*-Kaurene biosynthesis is enhanced by long photoperiods in the long-day plants *Spinacia oleracea* L. and *Agrostemma githago* L. Plant Physiol **101:** 25–29

**Zeevaart JAD, Gage DA, Talon M** (1993) Gibberellin A$_1$ is required for stem elongation in spinach. Proc Natl Acad Sci USA **90:** 7401–7405